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Identification of Differently Regulated Proteins after *Fusarium graminearum* Infection of Emmer (*Triticum dicoccum*) at Several Grain Ripening Stages

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Summary

This study was conducted to improve the knowledge of molecular processes involved in the interaction between *Fusarium graminearum* and emmer in the course of grain ripening. Emmer plants were artificially inoculated with a *F. graminearum* spore suspension at anthesis. In the course of grain ripening from milk ripe to plant death stage, grains at four phenological growth stages were collected for analysis. The infection degree was evaluated based on the *F. graminearum* DNA content in emmer grain infolding tissues (glumes and rachis). For proteome analysis the albumin and globulin fractions of emmer grains, consisting of proteins with various functions related to the development and stress response, were analysed regarding the changes due to *Fusarium* infection by two-dimensional gel electrophoresis. Altogether, forty-three proteins affected by infection were identified by mass spectrometry. Enzymes detoxifying reactive oxygen species were regulated at all developmental stages. In the early stage of grain development, the abundance of proteins related to stress response, such as 2-Cys peroxiredoxin, a chitinase, a xylanase inhibitor and a spermidine synthase was increased. During later stage of grain development, the abundance of stress-related proteins, such as chitinases, heat shock proteins and an α-amylase inhibitor-like protein, decreased. During all ripening stages, but especially during medium milk stage (BBCH 75) and soft dough stage (BBCH 85), the abundance of proteins related to carbon metabolism, starch and protein biosynthesis as well as photosynthesis increased due to *F. graminearum* infection. At the plant death stage (BBCH 97) the abundance of only two proteins related to metabolism decreased.

Key words: *Fusarium* head blight, grain development, emmer, plant proteomics, defence proteins

Introduction

Fusarium head blight (FHB) is a cereal disease causing significant yield losses and in particular accumulation of several mycotoxins, such as trichothecenes deoxynivalenol (DON), nivalenol (NIV), their acetylated derivatives and zearalenone (ZEA) (*1*). The predominant species infesting cereals in Europe are *Fusarium graminearum* Schwabe (*Gibberella zeae* Schwein. Petch.) and *Fusarium*

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culmorum (W.G. Smith). The most critical period for infection and colonisation of cereal ears with *Fusarium* spp. is during the anthesis and the first half of the grain filling stage (*2*). Multiple mechanisms of defence and resistance are known to exist in plants. Two main types of resistance to FHB are widely recognized: type I resistance to initial infection and type II resistance to fungal spread within adjacent tissues. Furthermore, resistance against *Fusarium* infection involves the ability to degrade trichothecenes and the inhibition of trichothecene biosynthesis of the pathogen (*3*). An effective method to control FHB is the cultivation of resistant cultivars (*4*). Therefore, more information about infection mechanisms of the pathogen and respective defence strategies of the plants is needed.

Emmer is an ancient tetraploid crop with hulled grain. The domestication of emmer was the first step towards the evolution of free-threshing tetraploid durum wheat and hexaploid bread wheat. Emmer cultivars are supposed to be resistant to fungal diseases such as stem rust, prevalent in wet areas. Some cultivars are tolerant of heat and drought stress. Thus, emmer represents a useful genetic resource for resistance breeding in wheat concerning biotic and abiotic stresses (*5*).

So far, a few studies have been available concerning the sources of FHB resistance in emmer (*6,7*). Oliver *et al.* (*7*) found a wide variation in the susceptibility of emmer to *F. graminearum*, with varieties ranging from highly resistant to highly susceptible. A study of Buerstmayr *et al.* (*6*) on wild emmer cultivars from Israel showed that most of the plants were highly susceptible to *F. graminearum*. Nevertheless, occasionally genotypes showing lower infection rates have been found, which may serve as a source for resistance breeding in emmer and wheat.

Several proteomic studies have been performed, analysing various proteins with a potential role in plant-fungus interaction. These studies provide an insight into pathogenicity and host resistance against *Fusarium* spp. infection of cereals. Most of them focussed on the initial infection of wheat spikes (*8–10*) and barley spikes (*11,12*) during the first days after inoculation at anthesis. According to these studies up to three days after inoculation the abundance of many proteins related to carbon metabolism, photosynthesis, oxidative stress and fungal cell wall degradation was affected by *F. graminearum* infection of the spikes. Regarding the proteins involved in carbon metabolism and photosynthesis, controversial results concerning changes of abundances were described. Wang *et al.* (*8*) found that abundances of most of these proteins decreased after infection of wheat spikes, whereas Zhou *et al.* (*9*), Shin *et al.* (*10*) and Yang *et al.* (*11*) ascertained an increased energy metabolism in infected barley and wheat heads. Furthermore, pathogenesis-related proteins, such as chitinases and thaumatin-like proteins, as well as proteins involved in oxidative stress response were predominantly accumulated during the early infection of wheat and barley (*9–11*).

To our knowledge, little research has been carried out considering the differential expression of proteins in response to *Fusarium* infection at later stages of infection in the course of grain ripening. Dornez *et al.* (*13*) analysed wheat kernels 5, 15 and 25 days post anthesis (water ripe, milk ripe and soft dough stage) and inoculation with *F. graminearum* focussing on xylanase inhibitor proteins (XIP). Additionally, the abundance of several pathogenesis-related (PR) proteins, such as peroxidases and chitinases predominantly increased and of thaumatin-like proteins as well as a wheatwin-2 precursor decreased (*13*). Earlier studies investigated the proteome of mature emmer and naked barley grains long after *Fusarium* infection (*14,15*). These studies also revealed an increase in stress-related proteins, such as serine protease inhibitor and thaumatin-like protein, and decrease in proteins related to oxidative stress as well as chitinase in emmer. In naked barley increased abundance of transcriptional regulatory proteins and protease inhibitors was detected. Furthermore, the abundance of proteins involved in starch synthesis decreased in both varieties (*14,15*).

In the current study, changes of specific proteins due to *F. graminearum* infection were investigated in four grain development stages from milk ripe to plant death of emmer. The aim of this work is to investigate *F. graminearum* infection-induced changes in protein expression in emmer grain and to elucidate how these changes depend on the ripening stage and the progress of infection. Albumins and globulins possess multiple functions in growth, development and stress response of cereals (*16*). The investigation therefore focused on albumin and globulin protein fractions.

Materials and Methods

Experimental design

The field trial was carried out at Marienstein (Nörten-Hardenberg), near Göttingen, Germany, in 2011 as block design with eight plots of three to six meters with the emmer genotype Linie 9-102 (IPK Gatersleben, Leibniz Institute of Plant Genetics and Crop Plant Research, Stadt Seeland, Germany). The seeding rate was adjusted for a density of 280 germinable grains per m^2. Four plots were artificially spray-inoculated with *Fusarium graminearum* spore suspension (10^5 spores per mL; 50 mL per m^2) during flowering using a knapsack sprayer. The minimum space between artificially inoculated plots and non-inoculated plots (referred to as control) was five meters. Three predominant DON-producing strains of *F. graminearum* (FG 142, FG 143 and FG 144) were used for conidiospore production according to previous studies (*14,15*). The strains were isolated from wheat spikes in Bavaria and are reference material from the Division of Plant Pathology and Crop Protection at the Department of Crop Science of the Georg-August-University of Göttingen, Germany. The strains were cultured as a mixture of equal proportions on an autoclaved wheat straw suspension, consisting of 9 g of straw (ground to 1.5 mm size), 500 mL of distilled water and 50 mg of streptomycin sulphate, for ten days at 20 °C. The spore density was quantified with a Fuchs-Rosenthal chamber (0.0625 mm^2, depth 0.2 mm; Hausser Scientific, PA, Horsham, USA). Fifty ears from each plot were randomly sampled by use of scissors at the development stages BBCH 75, 85, 87 and 97 as documented in Table 1. The samples were freeze dried and stored at −80 °C until sample preparation. The development stages were identified according to the BBCH-scale (*17*). The BBCH-scale defines the phenological growth stages with

Table 1. Phenological growth stages of emmer plants and the corresponding days after inoculation (anthesis: 0 days after inoculation)

Phenological growth stages (BBCH code)	Days after inoculation
Medium milk (75)	19
Soft dough (85)	25
Hard dough (87)	32
Plant death and collapsing (97)	39

BBCH=Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (Federal Institute of Biology, Federal Plant Variety Office and Chemical Industry), Germany

a standardised decimal code. The abbreviation BBCH derives from Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (Federal Institute of Biology, Federal Plant Variety Office and Chemical Industry), Germany.

Sample preparation and protein extraction

For proteomics analysis, grains were manually removed from the ears and milled with a ball mill (Retsch®; Mixer Mill MM 400, Haan, Germany). Grain samples were stored at −80 °C prior to analysis. Albumins and globulins were extracted with 50 mM sodium phosphate buffer (pH=7.8), containing 0.1 M NaCl and 0.2 % protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). A mass of 100 mg of flour was extracted with 1 mL of sodium phosphate buffer and stirred for 2 h at 4 °C. After centrifugation at 8000×*g* and 4 °C for 10 min, 500 μL of the supernatant were transferred into another tube. A volume of 1.5 mL of ice-cold trichloroacetic acid (TCA) in acetone was added to the supernatants and the mixture was stored at –20 °C overnight to precipitate the protein. The cold samples were centrifuged (8000×*g* at 4 °C for 10 min) and the supernatants were discarded. The pellets were rinsed three times with cold acetone under stirring for 10 min at 4 °C and then centrifuged as before. The pellets were dried at 100 mbar for 10 min in a vacuum concentrator (RVC 2–25 CD, Christ GmbH, Osterode am Harz, Germany) and resuspended in 500 μL of lysis buffer (6 M urea, 2 M thiourea and 0.2 % Pharmalyte buffer; BioRad, Munich, Germany), pH=3–10, 2 % CHAPS (Carl Roth, Karlsruhe, Germany), 2 % dithiothreitol, 0.2 % protease inhibitor cocktail (Sigma-Aldrich) and 0.002 % Bromophenol Blue. For solubilisation of the protein, samples were shaken for 1 h at 33 °C in a Thermomixer (Eppendorf, Hamburg, Germany). Finally, protein concentrations were determined with a 2-D Quant Kit (GE Healthcare Life Sciences, Freiburg, Germany) and adjusted to a concentration of 1 μg per μL.

Fusarium graminearum DNA

Total DNA was extracted from 100 mg of grain hull tissue after removing the kernels according to a CTAB protocol described by Brandfass and Karlovsky (*18*). The content of *F. graminearum* DNA was determined by species-specific real-time PCR with a standard prepared from a pure *F. graminearum* culture and quantified by densitometry (*19*).

Quantitative LC-MS/MS of DON

Grain samples were analysed by high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS), as described by Adejumo *et al.* (*20*) in the laboratory of Molecular Phytopathology and Mycotoxin Research, Department of Crop Sciences, Georg-August-University of Göttingen, Germany.

Two-dimensional gel electrophoresis

For isoelectric focusing (IEF), commercially available immobilized pH gradient (IPG) strips (pH=3–10, 17 cm, BioRad) were used. A volume of 300 μL of the protein sample was loaded into a tray. The IPG strips were rehydrated overnight, covered with 1 mL of mineral oil to prevent evaporation. The IEF was performed in the Protean® IEF cell (BioRad) under the following conditions: 15 min at 0–250 V, 3 h at 250–10 000 V and 10 000–60 000 V for hours. The rapid ramp was chosen, the current was set to 50 μA per gel and the temperature was 20 °C. Afterwards, the IPG strips were incubated in two buffer agents for 15 min: the first solution contained 6 M urea, 2 % SDS, 0.375 M Tris-HCL (pH=8.8), 20 % glycerol and 2 % DTT, and the second solution contained 6 M urea, 2 % SDS, 0.375 M Tris-HCL (pH=8.8), 20 % glycerol and 2.5 % iodoacetamide. Finally, the strips were rinsed with SDS-PAGE running buffer (25 mM Tris, 192 mM glycine and 0.1 % SDS). SDS-PAGE was performed in the Protean® II xi Cell (BioRad) in 12 % polyacrylamide gels (20 cm×20 cm×1 mm) with a current of 30 mA per gel. The staining was performed with a modified colloidal Coomassie G-250 staining (blue silver) (*21*).

Data analysis

The gels were scanned with an image scanner (Epson Expression™ 10000 XL; Epson, Long Beach, CA, USA) using the LabScan v. 6.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The gel images were saved as TIF files and analysed using the PDQuest Basic v. 8.0.1 analysis software (BioRad). As required for statistical analysis, four biological replications of each group, artificial inoculation and control were used to create 'replicate groups' for each grain development stage. Spot detection and matching were carried out by using the automated 'spot detection wizard'. Spots that were present in at least three of the group members were added to the analysis set. The spot quantities in four control samples were compared with the spot quantities in four inoculated samples. For statistical analysis sets, the student's *t*-test with significance level of 90 % was chosen. The standard deviation between four replicate groups was under 50 %. Protein spots were accepted to change after *F. graminearum* infection if the difference between the mean values of control and artificially inoculated samples was higher than the factor of two or if a protein spot exclusively appeared in one group.

Protein identification by mass spectrometry

Tryptic digestion of proteins and identification of proteins by mass spectrometry (MS) were performed as described by Klodmann *et al.* (*22*). Procedures were based on peptide separation using the EASY-nLC System (Proxeon; Thermo Scientific, Bremen, Germany) and coupled

MS analyses using the MicrOTOF-Q II mass spectrometer (Bruker, Bremen, Germany). MS data evaluation was carried out using ProteinScape v. 2.1 software (Bruker) and the Mascot search algorithm (Matrix Science, London, UK) against: (*i*) the NCBI protein database (*23*), (*ii*) the UniProt protein database (*24*) and (*iii*) the *Arabidopsi*s protein database (*25*). The following Mascot search parameters were used: enzyme: trypsin/P (up to one missed cleavage allowed); global modification: carbamidomethylation (C), variable modifications: acetyl (N), oxidation (M); precursor ion mass tolerance: 15 ppm; fragment ion mass tolerance: 0.6 Da; peptide charge: 1+, 2+, and 3+; instrument type: electrospray ionization quadrupole time of flight. Minimum ion score was 30, minimum peptide length was four amino acids, significance threshold was set to 0.05 and protein and peptide assessments were carried out if the Mascot score was greater than 30 for proteins and 20 for peptides.

Results and Discussion

In this study we inoculated emmer grains with a spore suspension of *Fusarium graminearum* at anthesis. The regulation of specific proteins due to *Fusarium* inoculation compared to a non-inoculated control was investigated in four ripening stages of the grains by proteomic analysis.

Fusarium DNA and DON content

Table 2 shows the content of *F. graminearum* DNA and DON at different ripening stages of emmer grains. In the samples, the *F. graminearum* DNA content was below the limit of quantification (LOQ). After artificial inoculation, 4.1 mg of *F. graminearum* DNA per kg of dry matter was detected already at BBCH 75. The DNA amount increased up to 21.3 mg/kg at BBCH 97. DON contents were significantly increased in emmer grains after artificial inoculation (Table 2). Control grains showed no detectable DON. The significantly higher amount of *F. graminearum* DNA and DON content in inoculated plants as compared to control plants showed that the inoculation was successful.

Proteome analysis

After artificial *F. graminearum* infection a total of 52 proteins showed different expression patterns regarding all development stages (Fig. 1). At BBCH 75 and 85 the abundance of eight proteins increased due to *Fusarium* infection, whereas at BBCH 87, that of six proteins and at BBCH 97 only of three proteins increased (Fig. 1). On the contrary, no proteins were detected to be reduced at BBCH 75, whereas six proteins showed reduced expression at BBCH 85. The abundance of nine proteins decreased at BBCH 87 as well as of 12 proteins at BBCH 97 (Fig. 1). In summary, during earlier development stages, protein expression rather increased due to artificial *F. graminearum* infection, whereas proteins were predominantly reduced at later grain ripening stages. Proteins could be identified by LC-MS according to their peptide sequences after trypsin digestion and database search.

Oxidative burst-related proteins

In all development stages the abundance of proteins related to oxidative stress changed due to *Fusarium* infection. Table 3 shows changes of enzymes related to oxidative stress after *Fusarium* inoculation. Altogether, three antioxidative proteins were identified: a peroxidase, 2-Cys peroxiredoxin and a manganese superoxide dismutase. In the early grain development at BBCH 75, peroxidase 1 increased by a factor of 2.9, and by a factor of 6.3 at BBCH 87 in the inoculated grains compared to the control grains, whereupon the protein was exclusively expressed in the inoculated grains at BBCH 97. The 2-Cys peroxiredoxin increased in the early grain development at BBCH 75 and 85 and decreased at BBCH 87 and 97. Furthermore, the abundance of a manganese superoxide dismutase was found to decrease at BBCH 85.

The increased accumulation of antioxidant enzymes in the grains suggests an oxidative burst after the infection with *F. graminearum*. Plants produce high levels of reactive oxygen species (ROS) such as hydrogen peroxide as a response to biotic or abiotic stress (*26*). Part of defence against biotrophic pathogens, oxidative burst contributes to the hypersensitive response and consequently to cell death at the site of the pathogen infection, limiting the pathogen spread to adjacent tissues. On the other hand, the plants produce antioxidants and ROS-scavenging enzymes to detoxify these reactive molecules (*27*). ROS are also involved in cellular signalling pathways associated with the induction of defence responses. Their regulation by antioxidative enzymes is proposed to play an important role in plant defence, since the ROS levels during oxidative burst mediate complex cell signalling networks (*26,28*). Peroxidases are furthermore involved in lignin-polysaccharide cross linking in plant cell walls, leading to higher resistance of the cell wall against enzy-

Table 2. *Fusarium* DNA content of emmer grain infolding tissues (glumes and rachis) and deoxynivalenol (DON) content of grains during ripening

BBCH	Control		Inoculation	
	w(*Fusarium* DNA)	*w*(DON)	*w*(Fusarium DNA)	*w*(DON)
	mg/kg	mg/kg	mg/kg	mg/kg
75	<LOQ	<LOD	4.1±1.6	15.0±7.3
85	<LOQ	<LOD	7.4±3.4	18.6±5.6
87	<LOQ	<LOD	7.6±3.8	14.9±3.2
97	<LOQ	<LOD	21.3±9.7	15.9±2.8

The values are expressed as mean±standard deviation. BBCH=see Table 1, LOQ=limit of quantification (0.02 mg/kg), LOD=limit of detection

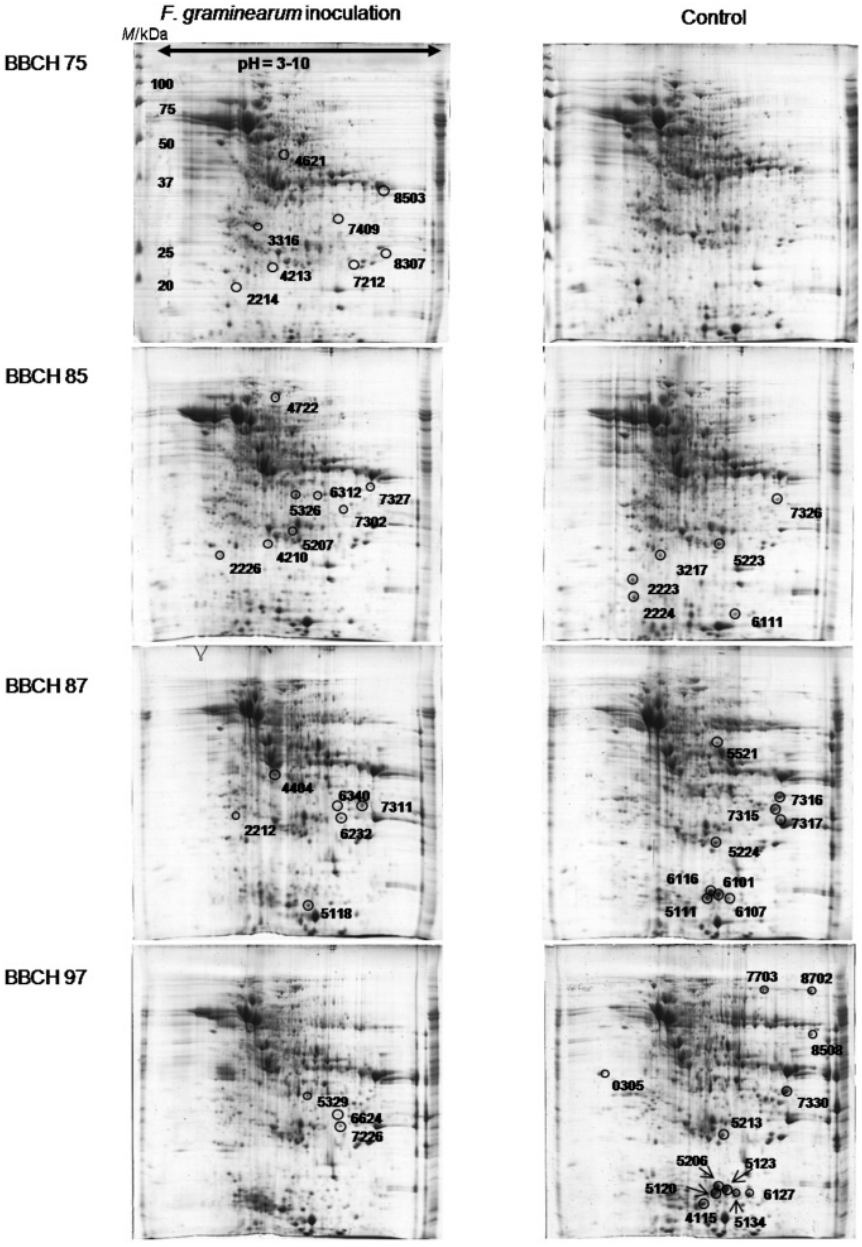

Fig. 1. Two-dimensional protein gels of emmer grains (12 % SDS-PAGE, immobilized pH gradient: pH=3–10, 17 cm linear strip, Coomassie G250-stained) at four development stages (BBCH 75, 85, 87 and 97, see Table 1) inoculated with *F. graminearum* and from control plots (see Materials and Methods). Spots of changed volume are encircled and numbered with IDs. BBCH=Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (Federal Institute of Biology, Federal Plant Variety Office and Chemical Industry), Germany

Table 3. Changes in the abundance of proteins related to oxidative burst due to artificial *F. graminearum* inoculation compared to control during grain ripening stages of emmer

Protein	BBCH stage			
	75	85	87	97
Peroxidase 1	+2.9		+6.3	+∞
2-Cys peroxiredoxin BAS1	+∞	+3.1	–3.7	–10.8
Manganese superoxide dismutase		–4.2		

BBCH=see Table 1, +=increased abundance, –=decreased abundance, +∞=protein found only in artificially inoculated grains

matic hydrolysis (*29*). In addition, ROS have been identified to induce DON biosynthesis in *F. graminearum*, whereas the presence of catalase reduced DON accumulation (*30*).

Pathogenesis-related proteins

An important plant strategy to inhibit fungal growth is the induction of PR proteins. These proteins are known to be induced in plants that are exposed to pathogens. They are assumed to protect plants against pathogenic microorganisms and various pests as well as abiotic stress (*31*). In the present study the abundance of PR protein levels in emmer grains changed after *Fusarium* infection (Table 4).

Table 4. Changes in the abundance of pathogenesis-related proteins due to artificial *F. graminearum* inoculation compared to control during grain ripening stages of emmer

Protein	Function	BBCH stage			
		75	85	87	97
Class II chitinase	Chitin catabolic process	+2.5		–10.5	
Basic endochitinase A (class I)	Chitin catabolic process			–12.5	–11.5
Predicted (chitin-binding) protein	Chitin binding			+3.6	+∞
Xylanase inhibitor XIP-III	Inhibition of fungal spread		+2.6		
Predicted (small heat shock) protein	Protein folding			–∞	
HSP 70 (heat shock protein)	Protein folding			–∞	
Hypothetical protein	Defence response		–3.5		
Globulin	α-amylase inhibitor			–8.1	

BBCH=see Table 1, +=increased abundance, –=decreased abundance, +∞=protein found only in artificially inoculated grains; –∞= protein found only in control grains

Several of these proteins belong to the group of chitinases. Chitinases catalyse the hydrolysis of chitin, a linear polymer of β-1,4-linked *N*-acetyl glucosamine. The amino acid sequences of chitinase subfamilies I and II are highly similar. The main difference is that class I chitinases possess a cysteine-rich chitin-binding domain, which has no catalytic function but is suggested to promote the catabolic activity of the enzymes (*32*). Besides pathogen infection, chitinase production depends on various biotic and abiotic stress factors, such as wounding, heavy metals, drought and cold stress. The enzymes protect plants directly by destroying fungal cell walls and indirectly by generating oligomers of chitin which act as signal molecules, including further defence responses (*32,33*). In the present study, the amount of chitinases predominantly decreased. Nevertheless, the amount of a class II endochitinase increased after *Fusarium* infection in the early grain development at BBCH 75 and later decreased at BBCH 87. Furthermore, the amount of a class I endochitinase decreased at BBCH 87 and 97. Interestingly, in the later stages of infection at BBCH 87 and 97 the amount of a 'predicted protein' containing a chitin-binding domain increased. Similarly, chitinase in the spikelets of barley was increased with *F. graminearum* infection three days after inoculation (*12*). Proteome analysis of mature emmer grains after *Fusarium* infection showed that the amount of class II chitinase was reduced. The authors suggested that a fungal signal was responsible for the effect (*14*). Lutz *et al.* (*34*) discovered that DON-producing *F. culmorum* and *F. graminearum* strains inhibit the expression of a chitinase gene from *Trichoderma atroviride*, demonstrating a possible fungal control of gene regulation concerning chitinase expression.

A xylanase inhibitor protein (XIP), playing a role in plant defence against fungi, increased at BBCH 85 with a factor of 2.6. These proteins belong to the glycosyl hydrolase 18 family, similar to chitinases. These proteins inhibit cell wall-degrading fungal xylanases and therefore inhibit pathogen spread. According to Dornez *et al.* (*13*), the abundance of some isoforms of xylanase inhibitor increased, whereas of others decreased since 5 days post anthesis. During grain ripening the abundance of most xylanase inhibitors increased.

Two heat shock proteins (HSP) decreased at BBCH 87. HSPs are known to assist in the correct folding of polypeptides as molecular chaperones and assist the refolding of non-native proteins. They are known to play a role in protecting plants from stress by securing correct protein conformation under stress conditions (*35*).

An α-amylase inhibitor decreased by a factor of 8.1 at BBCH 87. Alpha-amylase inhibitors protect the starch of the endosperm from fungal degradation. The abundance of most of the PR proteins decreased especially in the later stage of grain development starting with BBCH 87. It remains unclear whether the pathogen inhibited the formation of PR proteins or the abundance of PR proteins was reduced as a side-effect of the redirection of plant resources to other defence mechanisms such as cell-wall thickening and phytoalexin accumulation.

Energy metabolism, carbohydrate metabolism and photosynthesis-related proteins

A number of various proteins changed after *F. graminearum* infection are involved in energy and carbohydrate metabolisms, photosynthesis and starch and protein synthesis. Especially in the earlier ripening stages, the content of proteins involved in glycolysis, citric acid cycle and electron transport chain increased (Table 5). Zhou *et al.* (*9*) suggested a possible connection of glycolysis between *F. graminearum* and wheat to benefit the carbon assimilation of the fungus.

The amount of another protein, spermidine synthase, increased by a factor of 4.2 at BBCH 75. Spermidine synthase is involved in the biosynthesis of the polyamine spermidine. Polyamines occur in all living cells. They are involved in several cellular processes such as gene expression, translation, cell division and development as well as cell signalling (*36*). Polyamines are also involved in stress response and resistance to pathogen infection (*37*). It has been determined that some intermediates and products of the polyamine pathway, such as agmatine and putrescine, are strong inducers of TRI5 gene expression *in vitro* and therefore inducers of DON production (*38*). DON is known to be a virulence factor promoting fungal spread within wheat spikelets (*39*). Gardiner *et al.* (*40*) observed a significant increase in putrescine and spermidine in *F. graminearum*-infected wheat heads one to seven days after inoculation in comparison with a mock inoculation.

Furthermore, polyamines (putrescine, spermine and spermidine) are amongst other functions suggested to be

Table 5. Changes in the abundance of proteins related to energy and carbon metabolism and photosynthesis due to artificial *F. graminearum* inoculation compared to control during grain ripening stages of emmer

Protein	Function	BBCH stage			
		75	85	87	97
Glucose and ribitol dehydrogenase homologue	Carbohydrate metabolism	+2.8	+9.9		+5.9
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	+3.4			
Succinate dehydrogenase	Citric acid cycle, electron transport chain			+10.1	
Glucose-1-phosphate adenylyltransferase	Glycan/starch biosynthesis	+3			
Pyruvate, phosphate dikinase 1 (proteolytic fragment)	Photosynthesis	+5.4			
Ferredoxin-NADP reductase	Photosynthesis, energy metabolism		+6.6		
Eukaryotic translation initiation factor 6-2	Protein biosynthesis			+2,5	
60S acidic ribosomal protein P0	Protein biosynthesis			+4.9	
Spermidine synthase		+4.2			

BBCH=see Table 1, +=increased abundance, –=decreased abundance

involved in ROS-scavenging processes (*41*). In a study by Jang *et al.* (*42*) an increased polyamine biosynthesis prevented the accumulation of reactive oxygen species in rice. Additionally, an enhanced expression of ROS-detoxifying enzymes was found, associated with higher polyamine content.

At BBCH 85, 87 and 97 stages, the abundance of several predicted proteins with currently unknown functions decreased. Furthermore, some of the identified and unidentified proteins were apparently proteolytic fragments.

Conclusions

To our knowledge this is the first proteomic study analysing the effect of *F. graminearum* infection on cereal grains covering all ripening stages from early grain development until plant death. We found that inoculation of emmer with *F. graminearum* led to changes of protein expression in all development stages. In the early ripening stages, proteins predominantly related to metabolism and photosynthesis as well as stress-related proteins such as PR proteins and proteins related to oxidative stress were up-regulated. Additionally, a spermidine synthase was up-regulated at BBCH 75. During later ripening stages, at BBCH 87 and 97, the abundance of stress-related proteins decreased. Nevertheless, the abundance of some stress--related proteins, such as peroxidase and chitin-binding proteins, increased in after *F. graminearum* infection during later grain ripening stages, demonstrating that some defence strategies were persistent during the whole infection period. It is imaginable that the pathogen benefits from the enhanced metabolism, since plant metabolites serve as nutrients for fungal growth. The results identify molecular mechanisms initiated by *F. graminearum* infection of emmer grains. Further studies may compare susceptible and resistant emmer and wheat cultivars concerning their response to pathogen attack at different grain ripening stages to understand the response mechanisms of cereals during the entire infection period.

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